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(54) Title: HUMAN ERYTHROPOIETIN cDNA CLONES

(57) Abstract

A peptide immunochemically reactive with a monoclonal antibody to human erythropoietin, to a DNA fragment with a sequence coding for such peptide, to a recombinant DNA molecule having said DNA fragment inserted therein and to a transformed organism comprising an expression vector having said DNA molecule inserted therein, said organism being capable of producing said peptide, either directly or as a fusion protein. The present invention is also directed to methods for producing each of the above peptide, DNA fragment, DNA molecule and transformed organism.

Application Copy

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HUMAN ERYTHROPOIETIN cDNA CLONES

The United States Government has rights to this invention by virtue of grants No. RO1-HL21683 and No. RO1-HL30862 by the National Institute of Health, Bethesda, Maryland.

Field of the Invention

The present invention relates to cDNA clones of human erythropoietin (Ep), to methods of identification and preparation of such clones, and to their expression products. More particularly, the present invention relates to: (a) cDNA clones of human Ep identified from a cDNA-library constructed from human kidney mRNA, (b) synthesis of human Ep cDNA and insertion into pBR322 plasmids, (c) generation of E.coli expressing said cDNA, and (d) the products of expression of said cDNA.

The entire disclosure of my copending United States patent application Serial No. 570,075, filed on January 11, 1984 and entitled "REVERSE IMMUNOAFFINITY CHROMATOGRAPHY PURIFICATION METHOD" is hereby incorporated by reference as if fully set forth herein (said application being hereinafter referred to as the "Ep Purification Patent Application").

The entire disclosure of my copending United States patent application Serial No. 570,039, filed on January 11, 1984 and entitled "MONOCLONAL ANTIBODY TO HUMAN URINARY ERYTHROPOIETIN AND HYBRIDOMA SECRETING SAID ANTIBODY" is hereby incorporated by reference as if fully set forth herein (said application

being hereinafter referred to as the "Anti-Ep Patent Application"). Copies of extensive excerpts from both applications are attached as Appendix A and B, respectively.

Background of the Invention

A reliable and plentiful supply of human Ep has long been in demand to assist in a better understanding of the molecular mechanism of erythropoiesis and thus contribute to (a) the development of methods for the diagnosis and treatment of anemias and (b) the understanding of the differentiation and development of mammalian cells. Unavailability of sufficient human Ep has been due to scarcity of raw material, difficulties in its purification and lack of precise knowledge on the biogenesis of Ep.

The progress made by the present inventor in native human Ep purification (described in the Ep Purification Patent Application) by direct and reverse immunoaffinity chromatography, and in preparation of monoclonal Anti-Ep (described in the Anti-Ep Patent Application) has made it possible to attempt cloning of human Ep genes which, upon expression, can produce Ep protein.

Objects of the Invention

Accordingly, it is an object of this invention to provide an alternative source of human Ep protein.

Another object of this invention is to identify human Ep mRNA.

Yet another object of this invention is to synthesize and characterize human Ep cDNA.

Still another object of this invention is to produce a recombinant organism expressing human Ep cDNA.

A further object of this invention is to purify the product of said Ep cDNA expression.

A still further object of this invention is to characterize the sequence of human Ep cDNA and to identify the composition of human Ep.

These and other objects of this invention will be apparent to those skilled in the art in light of the present description, accompanying claims and appended drawings.

Summary of the Invention

One aspect of the present invention relates to a peptide immunochemically reactive with a monoclonal antibody to human erythropoietin.

Another aspect of the present invention relates to a DNA fragment coding for said peptide.

Yet another aspect of the present invention relates to a DNA molecule having inserted therein a DNA fragment comprising a DNA sequence coding for said peptide.

Still another aspect of this invention relates to a transformed living organism containing said DNA molecule said organism being capable of expressing said peptide.

Finally, this invention relates to methods for producing the above peptide, DNA fragment, DNA molecule and organism.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 is a fluorograph of electrophoresis of *in vitro* translation products of human kidney poly(A)$^+$ mRNA and immunoprecipitation of the translation products by monoclonal Anti-Ep.

Figure 2A depicts a silver stain of total proteins of human kidney extract, crude and pure Ep resolved by SDS-PAGE.

Figure 2B depicts an autoradiograph of an immunoblot from identical samples showing immunological detection of Ep-specific proteins.

Figure 3 is an autoradiograph of electrophoresis of the *in vitro* translation products of agarose gel size-fractionated mRNA.

Figure 4A is an autoradiograph of a nitrocellulose filter bearing positive colonies, as detected by colony hybridization.

Figure 4B is an autoradiograph of the same type of filter bearing a positive clone, pEp2, expressed in *E.coli* as a beta-lactamase fusion protein, detected by *in situ* radioimmunoassay using monoclonal Anti-Ep.

Figure 5 is a photograph of agarose gel electrophoresis of cDNA inserts for positive Ep clones, visualized by ethidium bromide staining and ultraviolet transillumination.

Figure 6A is a fluorograph of electrophoresis of in vitro translation products from mRNA selected by hybridization with positive Ep clones.

Figure 6B is an Anti-Ep immunoblot of electrophoresis of in vitro translation products of mRNA selected by hybridization with positive Ep clones.

Figure 7 is an autoradiograph of competitive immunoprecipitation of hybrid selected translation products of M.W. 29,000 and 15,000 in the absence or presence of unlabeled Ep.

Detailed Description of the Invention

In order to clone Ep cDNA, its functional mRNA is first isolated. Difficulties in isolating this mRNA stem from the fact that the specific cellular site of Ep synthesis has not been firmly established, although the kidney is known to play a key role. Additional difficulties arise from the scarcity of viable human kidney samples and from their low Ep levels. It was therefore necessary to search for sources with elevated Ep levels and greater tissue availability.

It has been well documented that erythrocytosis may be associated with various renal tumors. Tumor extracts from some renal carcinoma patients have shown an increased level of Ep and such tumors were often considered as Ep-producing tumors. However, renal carcinoma tumors are only rarely accompanied by significantly increased Ep levels. Thus, the ability of renal carcinoma tissue to produce Ep after explantation (and in culture) is by no means assured. Accordingly, the present inventor engaged in an extensive search for human renal tumors showing increased Ep levels, in an effort to identify and secure a continuous source of functional Ep mRNA.

In the work leading to the present invention, a total of thirty-six (36) renal cell carcinomas were examined. Two of those demonstrated very high levels of Ep (0.9 and 3 units/g of tissue) by exhypoxic polycythemic mouse bioassay. It was observed that both normal tissue and cancerous tissue explants of carcinoma-affected kidneys sometimes displayed elevated Ep titers. By contrast, tissue explants of normal kidneys showed no detectable Ep activity by the same assay.

Prior to the present invention, there had been no specific procedure for the isolation of human kidney mRNA. Renal tissue is very rich in mRNA-inactivating ribonucleases (RNase) and is extremely difficult to homogenize. Further difficulties arise because of the limited availability of nephrectomy samples. Accordingly, conventional fractionation of tissue samples (into cytoplasmic, microsomal and nuclear fractions) is impractical. Thus, total cellular RNA is preferably first isolated by extraction of disrupted kidney cells and messenger RNA is then selectively enriched, as follows:

Both normal and tumor portions from Ep-positive samples of renal carcinoma-affected tissues should be used for mRNA preparation. Throughout the procedure, care must be taken to minimize the degradative effect of (endogenous or exogenous) ribonuclease. Thus, preferably, RNase inhibitors are introduced in the extraction buffer. Reagents are sterilized and the glassware is baked at 250°C overnight prior to contact with RNA samples. Vanadyl-ribonucleoside (VRNS) complex or RNasin are preferred exogenous inhibitors of RNase, with VRNS being most preferred. Preferably, the extraction buffer also includes a combination of chaotropic agents (such as ethylene glycol, propylene glycol or other common glycols) or dissociating agents (e.g., sarkosyl, guanidine hydrochloride and guanidine isothiocyanate) and reducing agents (such as beta-mercaptoethanol). In general, the methods and reagents used significantly affect the yield and functionality of

the mRNA obtained. Most preferably, the extraction buffer contains guanidine isothiocyanate, sarkosyl, beta-mercaptoethanol, sodium citrate and vanadyl-ribonucleoside complex, which serve to disintegrate cellular structures, dissociate proteins and inactivate degradative enzymes and RNases.

The method of mRNA isolation employed in the present invention is fast, convenient and results in a good yield. The initial cell disruption is very important and it is preferably accomplished by dry-blending the tissue (as in an ordinary blender) in the presence of liquid nitrogen to ensure a sufficiently low temperature at which all enzymes will be inactive.

The powdered tissue is then treated with the extraction buffer. This is most conveniently and thoroughly accomplished in the powdering blender. Dissolution of the cellular material and shearing of the DNA takes place by further blending, and passing the sample through a needle. Shearing is evidenced by a significant drop in viscosity of the mixture.

The general procedure of Ulrich et al: Rat Insulin Gene: Construction of Plasmid Containing the Coding Sequences, Science 196: 1313 (1977), was used with specific modifications as described in Example 2.

The concentration of total RNA is measured by absorbance at 260nm. The absorbance ratios 260/230 and 260/280 are close to 280 are close to 2. This indicates no contamination of proteins and carbohydrates. A yield of about 0.5 to 1mg of RNA/gram of tissue is obtained.

Polyadenylated RNA (poly(A)$^+$ RNA) is then selected on an oligothymidylic acid-cellulose (oligo-dT cellulose) column as described by Aviv, H. et al, Purification of Biologically Active Globin Messenger RNA by Chromatography on Oligothymidylic Acid-Cellulose, Proc. Nat. Acad. Sci. (USA) 69:1408-1412 (1972). The poly(A)$^+$ RNA thus selected can be stored in ethanol at low temperature. Under these conditions, stability of the RNA is insured.

In order to confirm that the poly(A)$^+$ RNA contains Ep message (functional Ep mRNA) some of the oligo dT-cellulose-selected mRNA is translated in vitro. Translation may take place in a wheat germ cell-free system or, preferably, in a mRNA-dependent rabbit reticulocyte lysate system, as described by Pelham, H.R.B. et al, An Efficient mRNA-Dependent Translation System from Reticulocyte Lysates. Eur. J. Biochem. 67:247-256 (1976). The presence of mRNA coding for Ep protein in the poly(A)$^+$ kidney RNA is confirmed by immunoprecipitation of the translation products. Immunoprecipitation is carried out according to Kessler, S.W. Use of Protein-A Bearing Staphylococci for the Immune Precipitation and Isolation of Antigens from Cells: Meth. Enzymol. 73:442, 1981, with purified monoclonal Anti-Ep IgG designated as 7A7. This specific procedure is described in Example 4. Two polypeptides are immunoprecipitated with monoclonal Anti-Ep. Their molecular weights are 29,000 and 15,000 daltons, respectively (Fig. 1, lane 2). These polypeptides are not precipitated by preimmune mouse serum (Fig. 1, lane 3) nor are they detected in endogeneous translation and its immunoprecipitated sample (Fig.1, lanes 6 and 7). The size of these polypeptides is smaller than expected for native Ep. However, in a rabbit reticulocyte lysate system, where the ribosomes are free in the cytosol and where endoplasmic reticulum membrane organization, and membrane bound enzymes are absent, post-translational modification such as glycosylation is not expected to occur. Thus, the 29,000 dalton polypeptide which is precipitated specifically by Anti-Ep, may represent the aglycosylated form of Ep. The 15,000 dalton peptide may represent an immunologically related species. The presence of these polypeptides in tissue is also confirmed by immunoblotting of the tissue extract along with authentic Ep (Figure 2). The details of these experiments are described in Example 4.

Some of the thus obtained mRNA is enriched in Ep mRNA by size fractionation on agarose gel in the presence of CH_3HgOH according to Bailey, J.M. et al, Methylmercury as a Reversible Denaturing Agent for Agarose Gel Electrophoresis, Anal. Biochem. 70:75 (1976). The resolved RNA fractions are eluted and samples thereof are translated. The translation product is tested for Ep presence, preferably by immunoprecipitation with monoclonal Anti-Ep according to the method of Kessler, supra.

The fraction of agarose-gel-fractionated RNA that was enriched in Ep mRNA (fraction 11 in Fig. 3) is reverse-transcribed into [^{32}P]-labeled cDNA which is used as a hybridization probe in the identification of cDNA positive clones. Reverse transcription (using Avian myeloblastosis virus (AMV) reverse transcriptase) is preferable to T4 kinase RNA phosphorylation treatment because it gives a higher specific radioactivity (of the order of 10^7 cpm/ microgram) and it is less likely to label single-stranded contaminating ribosomal or tRNAs.

The remainder of poly(A)$^+$ kidney mRNA is used as the template for the synthesis of the 1st strand of cDNA by AMV reverse transcriptase in the presence of oligo-$dT_{(12-18)}$ as the primer. In view of: (a) the very rare opportunity presented in this case, i.e. the availability of a human kidney sample with significantly elevated Ep activity. and (b) the difficulties encountered in the preparation of intact polysomes from RNase-rich renal tissues, construction of a total human kidney cDNA library was undertaken. This approach not only entails minimum handling of the limited amounts of valuable RNA, but also allows other renal proteins of medical interest to be screened. Such a cDNA library would, of course, also require the screening of a large number of recombinants for Ep clones.

Conditions optimal for reverse transcription are highly dependent on the particular type of mRNA. The

purity of the reverse transcriptase, its RNase contamination, the ratio of reverse transcriptase activity to mRNA quantity, the substrate concentrations, the pH and ionic conditions of the reaction mixtures are all important factors which influence transcription efficiencies.

A ratio of 6 units of reverse transcriptase per microgram of mRNA template is preferred. Vanadyl ribonucleoside complex is preferably added to the transcription reaction mixture to inactivate contaminating RNase. The most preferred reaction conditions are set forth in Example 5. The general reverse transcription procedure is conducted according to Retzel, E.F. et al., Enzymatic Synthesis of DNA by the Avian Retrovirus Reverse Transcriptase in vitro, Biochem. 19:513-518, 1980.

At the end of the reaction period, template RNA is denatured preferably with CH_3HgOH to avoid interference of template mRNA in the synthesis of ds-cDNA.

Complementary cDNA is preferably synthesized using the large (Klenow) fragment of E.coli DNA polymerase I (which does not have the 5' --> 3' exonuclease activity) according to the method of Efstratiadis, A., et al., Enzymatic In Vitro Synthesis of Globin Genes, Cell, 7:279, 1976, wherein the reverse transcriptase loop from the previous step serves as the primer.

The hairpin loop covalently linking the first and second strand of cDNA is cleaved by nuclease S_1. The amount of S_1 required is determined by titration in pilot experiments using alkaline agarose gel electrophoresis, which permits the sizes of the DNA molecules to be visualized. The Klenow product behaves as a molecule twice the size of cDNA, and the blunt ended double stranded cDNA (ds cDNA) behaves as two molecules the size of the original cDNA strand. The optimum amount of S_1 nuclease was found to be 2.5 units per microgram of ds cDNA in this case.

In this work, 60 micrograms of poly(A)$^+$ RNA yielded 5 micrograms of blunt-ended ds cDNA.

The ds cDNA is inserted in the Pst I restriction endonuclease site (located in the ampicillin resistance gene) of pBR322 by homopolymeric dC:dG tailing according to Villa-Komaroff et al., A Bacterial Clone Synthesizing Proinsulin, Proc. Nat. Acad. Sci. (USA) 72:3727, 1978. The oligo(dC)-tailed cDNA and oligo(dG)-tailed vector are annealed preferably at a ratio of 1:2.

pBR322 is preferred because of its versatility as a plasmid cloning vector. It is under relaxed control and contains both ampicillin and tetracycline resistance genes. The Pst I cleavage site is a single cleavage site within the ampicillin resistance gene. Cloning into the Pst I site inactivates this gene. Thus, the clones will be ampicillin-sensitive. In addition, they should also be tetracycline-resistant, if they have taken up the plasmid.

The recombinant plasmids are transformed into E. coli C600 made competent to take up DNA by $CaCl_2$ and heat shock as described by Mandel, M. et al. Calcium Dependent Bacteriophage DNA Infection, J. Mol. Biol. 53:159, 1970.

Transformants are selected for tetracycline resistance and ampicillin sensitivity. Since, as mentioned above, the Pst I site is in the ampicillin resistance gene, transformants will be ampicillin-sensitive (Amp^S) but tetracycline-resistant (Tet^R).

E. coli C600 is preferably used as the recipient because it is a good host for large scale growth and purification of plasmids and can be transformed with high efficiency by use of a plasmid vector annealed to cDNA by dC:dG homopolymeric tailing.

For maximum efficiency of transformation, the bacterial culture should be in the logarithmic phase of growth and the cell density about 5×10^7 cell/ml at the time of treatment with calcium chloride. Maintaining the cells on ice for 12-24 hours prior to the transformation significantly increases transformation efficiency.

The optimum transformation reaction ratios are 1-10 ng of cDNA to 100 microliters of cell suspension. Larger amounts of suspension or cDNA result in lower transformation efficiencies. Use of top agar as a transformation plating medium is preferred.

Although there are several procedures for identification of recombinant plasmids that incorporate Ep DNA, the colonies are preferably initially screened by colony hybridization using [^{32}P] labeled cDNA synthesized from enriched Ep mRNA as the probe, followed by in situ colony radioimmunoassay (RIA) using purified monoclonal Anti-Ep IgG.

Colony hybridization is preferably conducted according to the general method of Grunstein, M. et al. A Method for the Isolation of Cloned DNAs that Contain a Specific Gene, Proc. Nat. Acad. Sci.(USA) 72:3961, 1975 here adapted for a smaller scale (in order to save materials, which are in short supply, while maintaining sensitivity of the screening procedure) by use of very small amounts of [^{32}P]-labeled cDNA as a probe with high specific radioactivity and small nitrocellulose filters, thus permitting a large number of colonies to be screened at a time. Duplicate colonies of transformants are grown on small filters, colonies are lysed and the DNA is denatured (by alkali treatment). Cell debris are treated with proteinase K and the DNA is fixed to the filter by baking. The DNA is hybridized to the probe, which binds only to its complementary DNA and permits positive colonies to be identified by autoradiography. This procedure is very efficient and results in elimination of about 95% of the colonies from further screening.

Positive colonies are picked and grown on nitrocellulose filters for further screening by in situ colony RIA. This method requires the expression of antigenic determinants, i.e., the expression of the cDNA inserted in pBR322 to produce a fused polypeptide containing the appropriate

antigenic site for Anti-Ep recognition. Given the availability of monoclonal Anti-Ep, RIA is a natural screening method choice for identification of positive clones. RIA is a particularly sensitive technique requiring only a small quantity of antigen containing an antigenic determinant. The general method of Helfman, D.M. et al: Identification of Clones that Encode Chicken Tropomyosin by Direct Immunological Screening of a cDNA Expression Library, Proc. Nat. Acad. Sci. (USA) 80:31 (1983) was employed.

Many operational variations of RIA are possible (direct or indirect, using ^{125}I-labeled-Anti-Ep or ^{125}I-labeled second antibody). The binding capacity and purity of the antibody significantly affect RIA sensitivity. Thus, use of crude ascitic fluid or only partially purified antibody should be avoided.

Radioiodination of antibodies is preferably carried out by the lactose peroxidase enzyme beads method as disclosed by Marchalonis, J.J., An Enzymic Method for the Trace Iodination of Immunoglobulins and Other Proteins, Biochem. J. 113:299, 1969.

In this work, three positive clones were identified and designated pEp1, 2 and 3. All three reacted consistently with monoclonal Anti-Ep 7A7 (both ^{125}I-labeled and unlabeled).

RIA-positive clones are grown in culture. Plasmid DNAs are isolated and the size of their cDNA inserts is determined by digestion with Pst I restriction endonuclease followed by electrophoresis in 6% polyacrylamide. Phage ØX174 RF DNA Hae III digested fragments are used as size markers. The sizes of the inserts are approximately 1400, 600 and 200 base pairs (bp) for pEp1, 2, and 3 respectively.

To further confirm that the positive clones indeed contain Ep sequences, hybridization selection of Ep-specific mRNA is performed as disclosed by Parnes,et al. Mouse beta-2 Microglobulin cDNA Clones: A Screening Procedure for cDNA Clones Corresponding to Rare mRNAs, Proc. Nat. Acad. Sci. (USA) 78:2253, 1981, modified as follows: Plasmid DNA is suspended in H_2O at 1mg/ml, heat denatured in 0.25 N NaOH, neutralized and spotted on nitrocellulose. The filters containing DNA are hybridized with poly(A)$^+$ RNA. It is advisable to use relatively large amounts of mRNA for clear results. (2.5 micrograms poly(A)$^+$ RNA/microgram of plasmid DNA).

Hybrid-selected mRNA is eluted from the filters, and translated in vitro as described above. ^{35}S-labeled translation products are analyzed by SDS-PAGE and fluorography. All three clones hybrid selected mRNA which directed the synthesis of polypeptides of molecular weight 15,000, 29,000, 66,000 and possibly 92,000 daltons. Final confirmation of the identity of the polypeptides was made by immunoblotting and competitive immunoprecipitation. SDS-PAGE of in vitro translation products from hybrid-selected mRNA are electrophoretically transferred onto a sheet of nitrocellulose paper (0.45 micron pore size) for immunoblotting with purified monoclonal Anti-Ep 7A7 IgG (obtained as described in the Anti-Ep patent application). This procedure, disclosed by Towbin, H. et al: Electrophoretic Transfer of Proteins from Polyacrylamide Gels to Nitrocellulose Sheet: Procedure and Some Applications, Proc. Nat. Acad. Sci. (USA) 76:4350 (1979), is described in detail in Example 9. Competitive immunoprecipitation is carried out in the presence of excess unlabeled pure Ep as given in Example 10. Of the hybrid selected in vitro synthesized polypeptides, the 29,000

dalton and 15,000 dalton polypeptides react with Anti-Ep on immunoblots and by immunoprecipitation and compete with authentic Ep for antibody binding.

The molecular weight of native human urinary Ep is 34,000 daltons. However, immunoblotting and immunoprecipitation of in vitro translation products of total kidney mRNA and hybrid selected mRNA all result in two polypeptides of M.W. 29,000 daltons and 15,000 daltons. Immunoprecipitation of tissue extracts from the Ep-rich renal carcinoma samples also resulted in identification of these two polypeptides, in addition to the 34,000 daltons polypeptide. The 34,000 daltons polypeptide is identical in size to the native glycosylated Ep. By contrast, the 29k polypeptide is smaller than expected for native glycosylated Ep. It is believed that in vitro translation in the message-dependent rabbit reticulocyte lysate system accounts for the smaller molecular weight, as discussed above. The hybrid selected 29,000 dalton peptide which was recognized specifically by monoclonal Anti-Ep is believed to be the aglycosylated form of Ep. The 15,000 dalton peptide, clearly immunologically related to, and sharing the antigenic determinants with, the 29,000 dalton and 34,000 dalton peptide, is believed to be an Ep-related protein.

Judging from the molecular weight of the native human urinary Ep, the cDNA insert of clone pEp1 is within the range of the coding size while those of clones pEp2 and pEp3 are too short to encode the complete sequence of Ep. These DNAs are useful in the preparation of full-length human Ep cDNA sequences for expression.

Alternatively, a cDNA fragment located at the 3' end of the mRNA, as determined by restriction mapping, will be used as a primer of mRNA reverse transcription. Since the 3' primer is a specific Ep cDNA fragment, the only cDNA synthesized by this reverse transcription reaction will be Ep cDNA. Optimization of reverse transcription is expected to yield entire Ep cDNA.

Sequencing will be performed according to the techniques described in detail by Maxam, A.M. et al, A New Method for Sequencing DNA, Proc. Nat. Acad. Sci. (USA) 74:560, 1977 and Sanger, F., et al., DNA Sequencing with Chain Terminating Inhibitors, Proc. Nat. Acad. Sci.(USA) 74:5463 (1977).

The following examples serve to illustrate the present invention without limiting its scope:

Materials and Sources:

agarose, and vanadyl-XTP's complex were from Bethesda Research Laboratories (BRL), Bethesda, MD.

creatine phosphate, spermidine, essential amino acids, Hepes, calf thymus DNA, lysozyme, tetracycline, ampicillin, dithiothreitol, ethylenediamine triacetate (EDTA), hemin, and sodium deoxycholate were from Sigma Chemical Co., St. Louis, Mo.

restriction endonucleases, pBR322 plasmid, E. coli DNA polymerase I, nuclease S_1, and phage ØX174 DNA-Hae III were from New England Biolabs, Beverly, Mass.

nitrocellulose filters were from Millipore, Bedford, Mass.

proteinase K, and calf liver tRNA were from Boehringer Mannheim Biochemical, Indianapolis, Indiana.

avian myeloblastosis virus (AMV) reverse transcriptase was from Life Sciences, Inc., St. Petersburg, Florida.

Sephadex G100, and protein A were from Pharmacia, Piscataway, N.J.

1,4-piperoxine-diethanesulfonic acid (PIPES), deionized formamide (dF), guanidine isothiocyanate, cesium chloride, and Sarkosyl were from Fluka Chemical Corp., Hauppauge, N.Y.

bovine serum albumin was from Schwartz-Mann Biochemical, Spring Valley, N.Y.

oligo-dT primer, and oligodithymidylic cellulose were from Collaborative Research, Waltham, Mass.

[^{35}S]-Methionine, ^{125}I-goat anti-mouse IgG, and lactose peroxidase enzyme beads were from New England Nuclear Company, Boston, Mass.

DNase was from Worthington, Freehold, N.J.

nitrocellulose sheet were from Schleicher & Schuell, Keene, N.H.

Triton X-100 (TM) detergent was from Eastman Kodak, Rochester, N.Y.

EXAMPLE 1:

Search for Human Renal Samples with High Ep Titer

Over a period of two and a half years, an extensive search was conducted for surgical renal carcinoma samples with Ep activity. Tissue from nephrectomy was obtained as fresh as possible, separated into normal and tumor portions, and washed with sterile, ice-cold phosphate-buffered saline to remove blood and extraneous material. Small portions were set aside for Ep bioassay and for the establishment of continuous culture. The remainder was frozen quickly in liquid nitrogen and stored at -70°C for the isolation of Ep mRNA. Ep activity in the tissue extracts was assayed in vivo by the exhypoxic polycythemic mouse method. Tissue homogenates were prepared by powdering the sample in liquid nitrogen and extracting it with 20 mM sodium phosphate, pH 7.8, (equal ratio, wt(g)/vol (ml)). The extract was then centrifuged at 30,000 x g for 30 minutes to remove cell debris and the clear supernatant was used for Ep bioassay.

Of 36 renal cell carcinoma extracts examined, two demonstrated high Ep activity (0.9 and 3.0 Ep units/ml),

six showed moderate activity (0.1 to 0.7 Ep units/ml) and the rest had either marginal (<0.1 Ep units/ml) or undetectable Ep activity. Tissue extracts of normal kidney from autopsy samples generally showed no detectable Ep activity but occasionally showed an activity of less than 0.05 units/ml. The increase in Ep activity in the renal carcinoma samples was found in both the normal and tumor portions of the kidney. The samples with high Ep bioactivity were used for mRNA preparation.

EXAMPLE 2:

Isolation and Purification Of Functional Human Kidney RNA

A nephrectomy sample from a patient suffering from renal carcinoma with erythrocytosis and elevated Ep activity (Ep positive) of 3.0 units/g of tissue, was selected from Example 1.

Both the normal and tumor portions of the renal tissues were (separately) used for mRNA preparation.

Total human kidney mRNA was isolated by the guanidine/ cesium chloride method of Ulrich et al, supra. RNA preparations were made from 10 g batches as needed. The frozen tissue was powdered in liquid nitrogen in a blender. 5 volumes of an extraction buffer were then added. The extraction buffer contained: 6M guanidine isothiocyanate/ 5mM sodium citrate pH 7.0/0.1 M beta-mercaptoethanol/0.5% Sarkosyl. The mixture was homogenized for an additional 3 min. DNA was sheared by passing the mixture through a 22 gauge needle. The solution was centrifuged at 10,000 rpm for 15 min and the supernatant was collected. Cesium chloride was added to 0.4 g/ml of solution and the resulting solution was layered onto a 5.7

M CsCl cushion containing 0.1 M EDTA (pH 7.5) (3 ml cushion and 2 ml of tissue extract) in a Beckman SW 50.1 centrifuge tube. Centrifugation was at 25,000 rpm for 16 hr at 20°C. The RNA pellet was dissolved in 10mM Tris-HCl, pH 7.4/5 mM EDTA/1% SDS, extracted with phenol, chloroform/1-butanol (4:1, v/v) and precipitated with ethanol.

EXAMPLE 3:

Selection of Poly(A)+ RNA

The total RNA of Example 2 was poly(A)$^+$ selected on an oligo-dT cellulose column (0.9 x 10cm; bed vol 6.4 ml). RNA was suspended in 10mM Tris-HCl, 0.5M NaCl buffer (pH 7.4) and loaded onto the column. Selectively adsorbed poly(A)$^+$ RNA was eluted with 10mM Tris-HCl, pH 7.4, containing 1mM EDTA and 0.1% SDS and precipitated with 2 vols of ethanol at -20°C for 24 hours. The pellet was washed with 70% ethanol, resuspended in water and stored at -20°C. Usually 0.5 to 1 mg of total RNA was obtained from each gram of renal tissue and about 20μg of poly(A)$^+$ RNA was obtained per mg of total RNA.

EXAMPLE 4:

In Vitro Translation and Immunoprecipition

In vitro translation was carried out in a message-dependent rabbit reticulocyte lysate system, using [^{35}S]-methionine as a label (New England Nuclear, 1236 Ci/mmol). The lysate was preabsorbed with purified IgG of monoclonal Anti-Ep (50μg/ml) and cleared with fixed Staphylococcus aureus bearing protein A (250μg/ml, IgGSorb, obtained from New England Enzyme Center, Boston, Mass.). The reaction mixture (25μl) contained 1 to 5μg/ml poly(A)$^+$ RNA, [^{35}S]-methionine (NEN) 55μCi, Hepes (pH 7.6) 20mM, KCl 80mM, $Mg(OAc)_2$ 1.3 mM, and reticulocyte lysate 10μl. Incubation was carried out at 37°C for 1 hr. The labeled translation products were immunoprecipitated with purified IgG of monoclonal antibody to human Ep. Immunoprecipitation was carried out according to

Kessler, supra, using 5 to 50 μg of IgG. The reaction components were pre-absorbed with mouse serum and the incubation was carried out in 10 mM Tris-HCl, pH 8.2/0.15 M NaCl at 4°C for 24 hours. The immune complex was collected by 100 to 200 μl/of a 10% suspension of fixed Staphylococcus aureus bearing protein A by incubation at 4°C for 1 hour. The precipitate was washed 6 times with 50 mM Tris-HCl, pH 7.4./0.15 M NaCl/1 mM EDTA/0.1% $NaDodSO_4$/1% Na deoxycholate/1% Triton X-100 (Buffer W) and suspended in gel sample buffer for electrophoretic analysis. The translation products and their immunoprecipitated products were resolved by SDA-PAGE and analyzed by fluorography (Figure 1). Two immunospecific polypeptides were identified, one migrating at about MW 29,000 and the other at about 15,000 daltons (lane 2). These polypeptides were not precipitated by mouse preimmune serum (lane 3) and they were not detected in endogeneous translation and in the immunoprecipitated sample of such translation (lanes 6 and 7). As discussed above, the M.W. 29,000 peptide which was precipitated specifically by the monoclonal Anti-Ep, may represent the aglycosylated form of Ep. The M.W. 15,000 peptide, clearly immunologically related, may represent a precursor or degradative fragment of the aglycosylated Ep. To verify the existence of these polypeptides in the original tissue extract, immunoblotting was conducted. Crude and purified human urinary Ep were also included on the same blot so as to provide a direct comparison of their immunospecificity. A single polypeptide of M.W. 34,000 was blotted from both the crude and purified Ep (Figure 2B, lanes 2 and 3), whereas three polypeptides were detected in the tissue extract, M.W. 34,000, 29,000 and 16,000 daltons. The 34,000 dalton polypeptide is identical in size to the authentic glycosylated Ep, and the smaller polypeptides are likely candidates for the aglycosylated precursor or degraded forms of Ep. The presence of these immunospecific poly-

peptides both in the tissue extract and in the in vitro translation products supports their identification as Ep-related forms and the presence of functional Ep mRNA. An identical set of unblotted gel samples was subjected to silver stain for analysis of their total proteins (Figure 2A), with respect to their Ep-specific proteins (Figure 2B). The immunospecificity of monoclonal 7A7 is self-evident. These results indicate that the monoclonal Anti-Ep recognizes native glycosylated Ep as well as aglycosylated Ep and its precursors and fragments.

The monoclonal antibody to human Ep used in this work was prepared as described in detail in the Anti-Ep patent application.

Purified IgG was used for all immunoprecipitation, immunoscreening, and immunoblotting reactions described in the present work.

EXAMPLE 5:

Synthesis and Cloning of Double Stranded cDNA

Poly(A)$^+$ kidney mRNA (20μg) from Example 3 was used as template for cDNA enzymatic synthesis. Avian myeloblastosis virus (AMV) reverse transcriptase was used to synthesize the first strand of cDNA in the presence of oligo-dT_{12-18} primer. The conditions described are for 500μl reaction mixture with 20μg of template. The reaction mixture contains poly(A)$^+$ RNA template, 50 mM Tris, pH 8.3, 10 mM $MgCl_2$, 100 mM KCl, 140μg/ml primer, 1mM CH_3HgOH to denature RNA, 30 mM beta-mercaptoethanol, 1 mM vanadyl sulfate ribonucleoside complex, 2 mM each of deoxyribonucleotides, and 120 units of reverse transcriptase. Incubation was at 42°C for 1 hour. Free nucleotides were removed by gel filtration over Sephadex G100. The RNA template was denatured by treatment with 12 mM CH_3HgOH. The second strand of cDNA was synthesized using the Klenow fragment of E. coli DNA Polymerase I. The reaction consisted of 100 mM Hepes, pH 6.8, 70mM KCl, 7mM $MgCl_2$, 10mM DTT, 22.5mM beta-mercaptoethanol, 0.5mM of

each deoxyribonucleotide, and 100 units of Klenow enzyme per 2 g of cDNA. The reaction was carried out at 15°C for 18 hours in a final volume of 1 ml. After phenol extraction, Sephadex G100 gel filtration and ethanol precipitation, the cDNA was treated with S_1 nuclease to cleave the hairpin loop at the 5' end of the second strand. The amount of S_1 required was titrated for each experiment in pilot reactions using alkaline agarose gel electrophoresis. Optimum concentation was found to be 2.5 units of S_1 nuclease per ng of double stranded cDNA in this case. The reaction mixture also contained 30mM NaAc, pH 4.6, 300mM NaCl, and 3mM $ZnSO_4$ and the incubation was 37°C for 1 hour.

The cDNA was then treated with terminal transferase and dCTP to add 10-15 residues to the 3' end. Pst I-digested pBR322 was similarly treated with terminal transferase and dGTP. The reaction mixture contains 140mM potassium cacodylate, pH 7.2, 0.5mM $CoCl_2$, 240mM dCTP or dGTP, 1.5 mg/ml BSA and 6,000 u/ml terminal transferase. The reaction was carried out at 25°C for 15 min. The cDNA was inserted into the Pst I site of pBR322 by homopolymeric dC:dG tailing. The oligo (dC)-tailed cDNA and oligo(dG)-tailed vector were annealed at a molar ratio of 1:2 at 42°C for 2 hours. The recombinant plasmids were transformed into E. coli strain C600 by $CaCl_2$ treatment and heat shock. Transformants were selected for tetracycline resistance (Tet^R) and ampicillin sensitivity (Amp^S).

One ml of overnight E.coli C600 culture was inoculated into 100ml of L broth in a 500 ml flask. The cells were grown at 37°C with vigorous shaking to a density of about $5x10^7$ cells/ml. The culture was chilled on ice for 10 min. and then centrifuged at 4,000xg for 10 min at 4°C. The supernatant was discarded and the cells were suspended in (1/5 of the original culture volume) ice-cold, sterile solution of 100mM $CaCl_2$ and 20mM sodium acetate,

pH 6.5. The resulting suspension was kept on ice for 20 min, then centrifuged again for 10 min. The supernatant was discarded and the cells were resuspended in (1/100 of the original culture volume) ice-cold sterile solution of 0.1M $CaCl_2$ and 20mM sodium acetate. The suspension was kept on ice for 20 hours.

To 100 μl of the cell suspension, 1-10 ng of vector were added in each of ten tubes. The mixture was left on ice for 10-30 minutes. 1 ml of LB broth was added to each tube and incubation was carried out at 37°C for one hour with shaking, to allow the bacteria to recover and to express antibiotic resistance.

Transformants were selected for tetracycline resistance (Tet^R) and ampicillin sensitivity (Amp^S) on LB-Tet plates (10g bacto-tryptone, 5g yeast extract, 5g NaCl, 3.5 ml (1M) NaOH and 15g agar per liter containing 25 μg/ml tetracycline), and on LB-Amp plates (containing 100 μg/ml of ampicillin, instead of tetracycline). Colonies began to appear 12-16 hours after onset of 37°C incubation. The transformation frequency was 5×10^5 transformants per microgram of cDNA on selection for tetracyclin resistance. Approximately 95% of the transformants were both tetracycline-resistant and ampicillin-sensitive.

EXAMPLE 6:

Enrichment of Ep-mRNA and Synthesis of [^{32}P] cDNA probe

Methylmercury Hydroxide Agarose Gel Electrophoresis

Poly(A)$^+$ RNA (50 μg) was fractionated by electrophoresis in a 1.5% agarose gel containing 12.5 mM CH_3HgOH using low melt agarose (BRL, Bethesda, MD), at 40 volts for 15 hours. Gel lanes containing size markers ØX174 Hae III digest and ribosomal RNA's of HeLa cells) were soaked in 0.5 M ammonium acetate and stained with ethidium bromide. Gel lanes containing poly (A)$^+$ were soaked in 100 mM DTT to allow renaturation of RNA. The lanes were sliced into 30 fractions and the RNA was extracted from these fractions by controlled microwave

heating and phenol extraction, followed by ethanol precipitation. Portions of the fractionated RNA's were translated in vitro and immunoprecipitation of the translation products was carried out as described in Example 4 to locate the fraction(s) enriched in Ep mRNA. The ^{35}S-labeled translation products and their immunoprecipitates were analyzed by SDS-PAGE. The majority of Ep mRNA was resolved in fraction number 11 (Figure 3, lane 11), as detected by immunoprecipitation of the translation products with Anti-Ep 7A7 (Figure 1, lanes (4) and (5)). Using ribosomal RNA and ØXDNA Hae III fragments as markers, fraction number 11 corresponds approximately to 1400 bp in size. This fraction was used to synthesize ^{32}P-labeled single-stranded cDNA according to Example 5. A specific radioactivity of 10^7 cpm/μg was obtained. The ^{32}P-labeled cDNA was used as a probe for the initial screening of recombinant plasmids.

EXAMPLE 7:

Initial Screening of the cDNA Library:
In Situ Colony Hybridization

Tet^R, Amp^S transformants from Example 5 were individually picked and grown on nitrocellulose filters (Millipore, 4.5 cm containing 100 gridded squares). 100 colonies were inoculated onto each of 12 filters, each colony within a grid square. Each filter was placed on the surface of an LB-Tet plate and incubated at 37°C for 20 hours. The transformants were screened by a modification of colony hybridization using ^{32}P-labeled cDNA synthesized from size-fractionated mRNA enriched in Ep message.

Filters bearing colonies were treated with 0.4 N NaOH, neutralized, and treated with proteinase K. The DNA was fixed to the filter by baking at 80°C in vacuo for 4 hours. Twelve filters were hybridized together in 3 ml of probe at 1 x 10^6 cpm/ml in 50% deionized formamide (DF) and 0.75M NaCl/75mM sodium citrate at 37°C for 24 hours. The filters were washed six times with 0.3M NaCl/30mM

sodium citrate at room temperature (40 min./wash), blotted dry, and exposed to film.

A typical filter bearing the colony hybridization results is shown in Figure 4A, and two classes of positive colonies were detected. One, comprising 0.1 to 0.4% of the total colonies, hybridized very strongly to the probe and exhibited dense spots on autoradiography. The second class, which consisted of 5% of the total colonies, hybridized to the probe in varying degrees but significantly less strongly than did the first class. The remainder of the colonies were negative. By this preliminary screening, about 95% of the transformants were eliminated from further screening.

EXAMPLE 8:

Immunological Screening of Colonies and Characteriztion of Plasmid DNA

Immunological screening was carried out by in situ colony radioimmunoassay (RIA) with monoclonal antibodies to human Ep using the general method of Helfman et al., supra. Bacterial colonies were grown on 4.5 cm nitrocellulose filters as described above, and lysed over $CHCl_3$ vapor for 30 minutes. Each filter was treated with 10 ml of lysis buffer in a Petri dish at room temperature overnight with gentle shaking. The lysis buffer contains 3% bovine serum albumin (BSA), 50 μg/ml lysozyme, 2μg/ml DNase in 50mM Tris HCl pH 7.4./150 mM NaCl (Tris/saline). The filter was rinsed thoroughly with Tris/ saline and incubated at room temperature for one hour with 5 ml of purified IgG 7A7 (1mg/ml) in Tris/saline/3% BSA. The filters were washed six times with the same buffer at 45 minutes per wash to remove nonspecifically adsorbed antibody. Bound antibody was detected by a one hour incubation with ^{125}I-labeled affinity purified goat anti-mouse IgG

(1 x 10^6 cpm/ml). The filters were washed extensively (six to eight washes) with buffer W, and analyzed by autoradiography. Positive clones were verified by direct colony RIA using ^{125}I-labeled monoclonal anti-Ep (2 x 10^6 cpm/ml). Radioiodination was carried out by the lactose peroxidase method. In all RIA's, a filter spotted with various amounts of purified Ep was included as a control for the specificity of the immunological detection of the antigen. In such controls, <1 ng of purfied Ep could be detected.

Positive recombinants from colony hybridization were picked, and grown on gridded nitrocellulose filters in a registered fashion for immunological screening by in situ colony RIA with 7A7. This procedure relies on expression of the cDNA inserted in the pBR322 beta-lactamase operon to produce a fused polypeptide containing the appropriate antigenic site for the Anti-Ep recognition. From 1.4 x 10^5 transformants, three positive clones were identified that reacted consistently with 7A7. These clones were designated pEp1, 2, and 3. A representative filter showing the detection of such a positive clone (pEp2) is seen in Figure 4B. The immuno-specificity of the positive clones was further confirmed by direct colony RIA using ^{125}I-labeled 7A7. All three clones reacted positively and consistently with [^{125}I]7A7. The plasmid DNA of these clones was isolated and its size determined, as described below.

Plasmid DNA was prepared by the alkaline lysis procedure of Birnboim, H.C. et al, A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA, Nuc. Acid Res. 7:1513, 1979, and purified by centrifugation through 1M NaCl in a SW 50.1 rotor at 40,000 rpm for 6 hours at 20°C. Digestion with restriction endonucleases was carried out under conditions recommended by the supplier. Gel electrophoresis was carried out in 6% polyacrylamide gels or in 1% agarose gel. ØX174 RF DNA -

Hae III digested fragments were used as markers. The sizes of the inserts are 1,400, 600 and 200 base pairs for pEp1, 2 and 3 respectively (Figure 5, lanes 2, 4 and 1 respectively).

EXAMPLE 9:

Hybridization Selection of mRNA and In Vitro Translation

Selection of mRNA was conducted according to the procedures modified from Parnes et al., supra. Plasmid DNA was suspended in H_2O at 1 mg/ml, denatured by heating 2 minutes at 96°C in 0.25 N NaOH, cooled quickly, neutralized with HCl, and spotted to saturation on nitrocellulose. The filters were baked for 2 hours at 90°C and then hybridized with human kidney poly(A)$^+$ RNA at a ratio of 2.5 μg poly(A)$^+$ RNA per ug of plasmid DNA. Hybridization was carried out at 50°C for 3 hours in a total volume of 100 μl containing 65% (v/v) DF/20 mM 1,4-piperoxine-diethansulfonic acid (PIPES), pH 6.4/0.2% SDS/ 0.4. M NaCl / 100 μg per ml calf liver tRNA. The hybrid selected mRNA was eluted with 200 μl of H_2O at 100°C for 90 seconds and then snap frozen in liquid nitrogen. The eluate was precipitated with ethanol using 10 to 20 μg of calf liver tRNA as a carrier. The hybrid selected mRNA was translated in vitro and the ^{35}S-labeled translation products were resolved by SDS-PAGE and analyzed by fluorography (Figure 6). As seen in Figure 6A the RNAs selected by pEp1, 2 and 3, all directed the synthesis of four ^{35}S-labeled polypeptides (lanes 4, 5 and 6 respectively). The molecular weight of these polypeptides relative to the gel markers were approximately 92,000, 66,000, 29,000 and 15,000 daltons. The 92,000 dalton band was also seen in the endogeneous translation (lane 0) and in the pBR322 selected sample (lane 1) but at a much lesser intensity. The 66,000, 29,000, and 15,000 dalton polypeptides were not detected in lanes (0) and (1), indicating that they are Ep-specific.

Final confirmation of the identity of these polypeptides was made by immunoblotting and competitive immunoprecipitation as described in Example 10.

EXAMPLE 10:

Detection of Antigenic Proteins Synthesized in vitro by Immunoblotting, and Competitive Immunoprecipitation

Electrophoretic transfer of proteins was carried out according to the general procedure of Towbin et al., supra. Nitrocellulose, 0.45 μm pore size was used. The transfer was conducted in 25mM Tris HCl pH 8.4/192 mM glycine /20% methanol (v/v) at 0.35 amperes for 12 hours and then at 1 ampere for 3 hours using a Hoefer TE42 Transphor unit with a TE 50 power supply. The electrophoretic blot was rinsed with Tris/saline and incubated with 3% BSA/Tris/saline for 1 hour at 40°C to saturate the remaining protein binding sites. It was then incubated with 7A7 (1 mg/ml) in 3% BSA/Tris/saline for 1 hour at room temperature or overnight at 4°C. After washing with Tris/saline, the blot was incubated with ^{125}I-labeled goat anti-mouse IgG (1.2×10^6 cpm/ml) for 1 hour at room temperature. For every 100 cm^2 blot, 10 ml of the antibody solution was used. The blot was thoroughly washed with buffer W six to ten times at 45 minutes per wash and exposed to film. The results are seen in Figure 6B. Two polypeptides, M.W. 29,000 and 15,000 were immuno-blotted by the Anti-Ep whereas the M.W. 92,000 and 66,000 polypeptides were not detected. This is true in the translation of total kidney poly(A)$^+$ RNA (lanes 2 and 3) as well as in the hybrid selected mRNA (lanes 4, 5 and 6). Whether this is due to inefficient transfer of high molecular weight proteins or a lack of antigenic recognition remains to be studied.

Competitive immunoprecipitation of ^{35}S-labeled, hybrid selected translation products was carried out by the addition of purified unlabeled native Ep to the immune reaction mixture. Both the ^{35}S-labeled M.W. 29,000 and

15,000 polypeptides were immunoprecipitated by 7A7 in the absence of unlabeled Ep (Figure 7, lane 3). Precipitation of these polypeptides was inhibited by the addition of excess unlabeled Ep (lanes 4 and 5). Preimmune mouse serum did not precipitate these polypeptides (lane 1) nor was any precipitation detected in endogeneous translation (lane 2). About 50% inhibition resulted in the presence of 2 μg of unlabeled purified Ep (lane 4) and over 90% of inhibition was observed upon the addition of 10 μg of purified unlabeled Ep (lane 5). These results indicate that of the hybrid selected translation products, the 29,000 and 15,000 dalton polypeptides are recognized by monoclonal Anti-Ep, 7A7 and that authentic Ep competes with them for antibody binding.

APPENDIX A

Ep Purification

According to the present method, unconcentrated or concentrated urine from severely anemic patients can be used as the raw material. Starting samples, are preferably first centrifuged to eliminate insoluble material and purified preferably by hydrophobic interaction chromatography (HIC), as described by Lee-Huang, S.: A New Preparative Method for the Isolation of Human Erythropoietin With Hydrophobic Interaction Chromatography, Blood 56:620-624, 1980, in order to remove the bulk of urinary contaminants and permit more efficient and repeated use of the immunoadsorbents. HIC involves processing of the raw material through a crosslinked neutral gel chromatographic column wherein the gel contains a hydrophobic group. Phenyl-Sepharose CL4B is particularly preferred because it provides a strong yet easily reversible binding with Ep. Octyl-Sepharose may also be used, but Ep elution therefrom is less complete. The specific activity of Ep obtained from this step depends on the potency of the starting material but generally ranges between about 115 and 250 units per mg of protein. The yield is usually about 80%. One unit of Ep is defined as the activity contained in 0.5 mg of the second International Reference Preparation of Human Urinary Erythropoietin (IRP) (obtained from the World Health Organization, International Laboratories of Biological Standards, Hampstead, London, England), or one-tenth of the contents of one ampule of this preparation.

The HIC-purified material can be used as the immunogen to raise antibodies to Ep (hereinafter designated as "Anti-Ep") and its common contaminating impurities (hereinafter designated as "Anti-I"). This can be conveniently performed in a single immunization using antibody-producing laboratory animals. The immunization is carried out in accordance with methods well known in the art and, in the case of Ep or other weak immunogens, it preferably includes several booster injections in addition to the

initial injection. Anti-Ep titers are determined by the *in vivo* exhypoxic polycythemic mouse bioassay described by Camiscoli, J.F. and Gordon, A.S.: *Bioassay and Standardization of Erythropoietin* in Gordon, A.S. (Ed.) *Regulation of Hematopoiesis*, Meredith Corp., New York, 1970 pp 370-396.

Polycythemia is induced in mice by hypobaric hypoxia. In order to keep a high protein concentration and thus stabilize the Ep activity, Ep samples for assay are made up in a buffered albumin solution. Samples are injected into mice posthypoxia, intraperitoneally. Ep activity is measured by its stimulation of ^{59}Fe incorporation in red blood cells. ^{59}Fe incorporation is determined in a gamma counter. The results are compared to those obtained using the second IRP from WHO. Anti-Ep titers are determined by assaying for ability to neutralize Ep-stimulated ^{59}Fe incorporation in red blood cells.

The immunized laboratory animals are then finally bled. Antisera from the bleedings after the last injection are isolated, assayed for anti-Ep titers, and purified by immunoaffinity chromatography to eliminate non-immunoglobulins. The rabbit antisera are processed through a Sepharose 4B column to which goat-(anti-rabbit) Igs have been covalently linked. The non-immunoglobulins are excluded from the column, while the specific Igs are eluted with, e.g., 3M sodium thiocyanate (NaSCN) or 0.2M acetic acid.

The thus obtained specific immunoglobulin preparation is treated to separate Anti-Ep from Anti-I. For this purpose, a highly purified Ep preparation is preferably used. However, the present invention does not require pure Ep for antibody preparation and/or separation. Partially purified Ep (or other partially purified antigen), prepared according to conventional methods, is adequate for carrying out the method of the present invention.

The antibody separation may be preferably accomplished by a new principle and procedure which employs reversible binding of antigen to a supporting matrix and

thus permits subsequent recovery of valuable Ep (or other antigen) after it is used in the Anti-Ep (or other antibody) purification, without substantial loss of activity.

The antibody separation procedure utilizes the fact that Wheat germ Lectin-Sepharose 4B (WGLS) columns coated with purified Ep have differential affinity for their biospecific and immunospecific ligands. The procedure involves four steps:

1. Purified Ep is bound to WGLS to produce a WGLS-Ep complex. Ep binds tightly to WGLS due to interaction of its N-acetyl-glucosaminyl residues with the wheat germ lectin.

2. The affinity purified rabbit immunoglobulins are processed through a WGLS-Ep column: Anti-Ep binds to the WGLS-Ep complex, while Anti-I does not, but is excluded in the effluent and set aside for further use. Of course, since the original purified Ep, that was used to coat the WGLS column in Step 1, was not homogeneous, its impurities will also be carried over to the WGLS column of Step 1 and, consequently a small fraction of the Anti-I will bind to the WGLS-Ep column of Step 1. This was the shortcoming of conventional immmunoaffinity techniques which the present invention has overcome, as will be described below. Anti-Ep bound to the WGLS-Ep complex is eluted and preferably processed again through a regenerated WGLS-Ep column to insure complete resolution of Anti-Ep/Anti-I immunoglobulins. The Anti-I-containing eluents from the first and the second separation are pooled and Anti-I are recovered therefrom.

3. Since the affinity between the constituents of the immune complex (Ep-(Anti-Ep)) is lower than the affinity between Ep and the sugar-lectin complex (WGLS-Ep), Anti-Ep from WGLS-Ep-(Anti-Ep) can be selectively eluted using a weak acid or a dissociation reagent. The ability of WGLS to bind Ep both at low pH and under dissociating conditions makes WGLS a useful adsorbent for Anti-Ep

purification and at the same time enables recovery of the valuable Ep (see step 4 below). The thus recovered Anti-Ep is separated from the eluent (e.g., by dialysis) lyophilized, and stored frozen for subsequent use.

4. Ep can be recovered from WGLS-Ep, once Anti-Ep has been eluted, by further elution, preferably with N-acetylglucosamine or N,N-diacetylchitobiose. This is not possible under conventional immunoaffinity procedures since, normally, the immunoadsorbent is irreversibly coupled to the supporting matrix and cannot be recovered. When the supply of the antigen (used as the immunoadsorbent) is limited, the recovery of such materials is a very valuable saving. Alternatively, the column of WGLS-Ep can be regenerated and can be reused.

The thus recovered Anti-Ep and Anti-I are separately covalently linked to CNBr-activated Sepharose 4B. The coupling procedure has been generally described by Axen, R. et al "Chemical Coupling of Peptides and Proteins to Polysaccharides by Means of Cyanogen Halides" Nature, 214:1302-1304, 1967. The Sepharose-(Anti-Ep) and Sepharose-(Anti I) so prepared are used in column form for the direct immunoaffinity chromatography (DIAC) and reversed immunoaffinity chromatography (RIAC) purification of Ep.

The Ep purified by HIC is further purified by DIAC on a Sepharose-(Anti-Ep) column. This purification results in exclusion of the majority of contaminants from the column, which are carried off in the effluent, while Ep is retained on the column. It is important to note, however, that at this stage some antibodies to some minor impurities will be present in the Sepharose-(Anti-Ep) column because of the lack of homogeneous Ep in the immunoaffinity purification of the Anti-Ep. This is the intrinsic limitation of any conventional direct immunoaffinity technique.

Ep from the Sepharose-(Anti-Ep) column is eluted with an appropriate buffer. Choice of buffer is important

in preserving Ep activity. For example, commonly used immune complex dissociating acidic buffers or chaotropic ions (such as glycine hydrochloride buffer or sodium thiocyanate) inactivate Ep, while simple alkali gives incomplete desorption. The present inventor has found that inclusion of 10-20% of a polarity reducing agent (such as glycerol or another common 1,2-glycol) and a dissociation agent (such as guanidine hydrochloride or urea) in an alkaline eluant (such as NaOH) facilitates effective release of Ep from the immunoadsorbent while preserving Ep activity. Preferred are ethylene glycol and guanidine hydrochloride, which can be easily removed and which appear to have no detrimental effect on Ep activity.

The thus eluted Ep is dialyzed (preferably immediately and thoroughly) against water and sodium phosphate buffer. Under these conditions, DIAC is very efficient, offering a high purification factor (usually about 169-fold over HIC) and a high yield (usually about 80% or higher). However, the main limitation of DIAC is the impurities in the original Ep preparation. The antibodies against these impurities are carried over in the purification system and immunoadsorb their antigens in the Sepharose-(Anti-Ep) column. As a consequence, the purity of the DIAC product cannot exceed that of the original Ep used in preparation of the WGLS column (Step 1) for antibody purification.

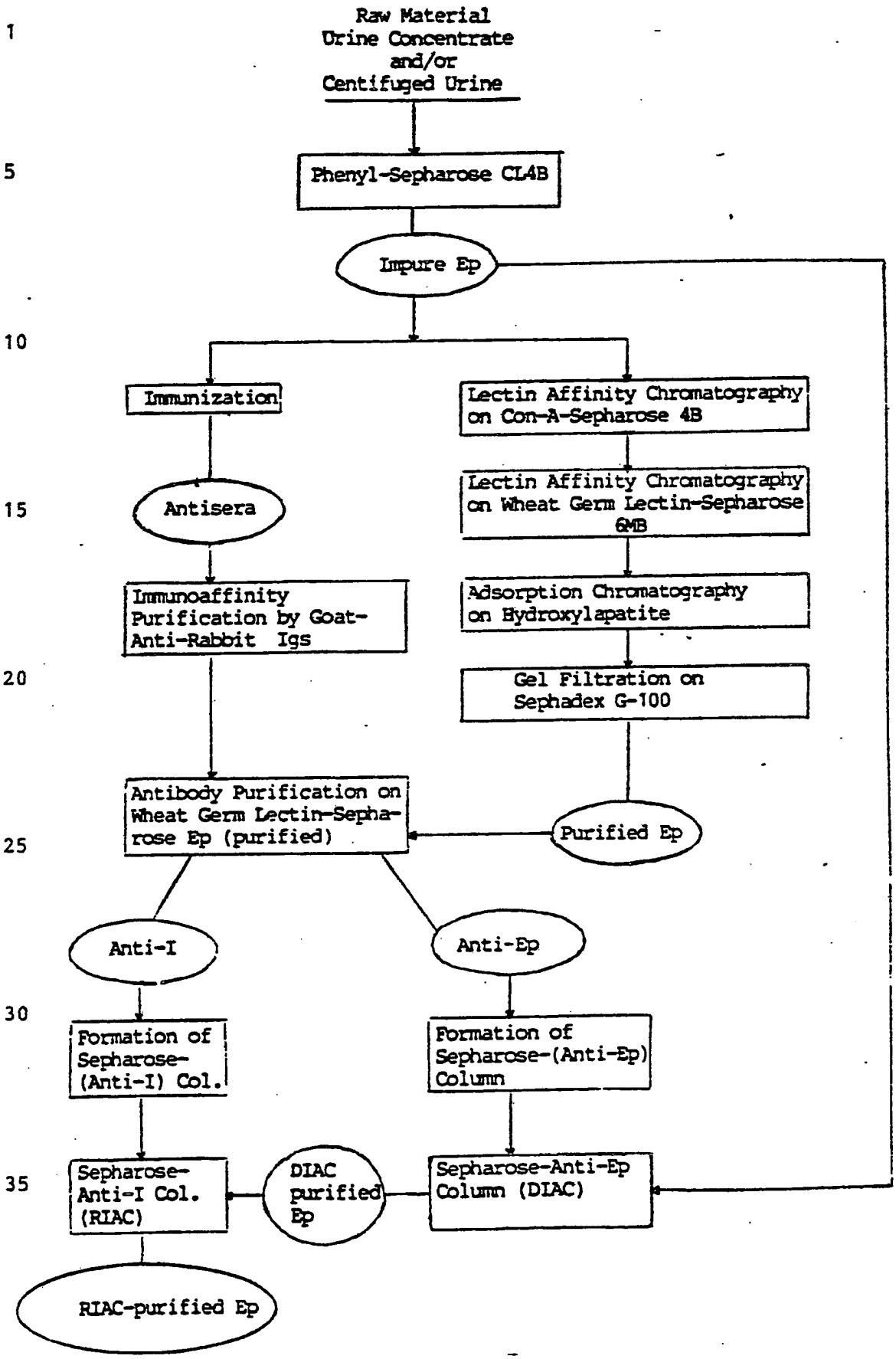
At this point further purification is accomplished with another Sepharose column coupled with Anti-I. While Anti-Ep contains only a minor fraction of antibodies to the impurities, Anti-I consists of the bulk of these antibodies. Thus, the Sepharose-(Anti-I) column will be able to provide sufficient antibody sites to bind substantially all the impurities contained in the DIAC-purified Ep.

Upon loading DIAC-purified Ep onto a Sepharose-(Anti-I) column, the trace impurities are retained in the column due to the formation of specific immune complexes with their corresponding antibodies, which are present in

great excess on the column, whereas pure Ep is selectively excluded in the effluent. This step affords preparation of Ep which is purer than the original antigen. Such efficiency is not attainable with other conventional immunoaffinity techniques. This immunoaffinity chromatography step wherein the impurities are bound to their antibodies, while the valuable protein is excluded in the effluent, is referred to as Reverse Immunoaffinity Chromatography (RIAC).

The impurities removed in the reverse immunoaffinity step are a constant set of residual urinary contaminants; and they have been copurified with Ep in many separation techniques, and are therefore fairly uniform from batch to batch. Thus, crude urine from a source different from that employed to generate the antisera can be effectively purified by the HIC-DIAC-RIAC procedure. The amount of Anti-I required for immunoadsorption of these minor impurities of DIAC-purified Ep is small relative to the total capacity of the Sepharose-(Anti I) column. Furthermore, since reverse immunoaffinity chromatography immunoadsorbs only the contaminating impurities, no desorption of Ep is required, thus minimizing manipulation of valuable samples and increasing yield accordingly. The impurities retained on the column can be subsequently dissociated from the immunoadsorbent by eluting with an appropriate acidic eluent. The column is thus regenerated and ready for subsequent use.

A flow chart outlining the various steps of the present invention is set forth on the following page.


Raw Material
Urine Concentrate
and/or
Centifuged Urine
Phenyl-Sepharose CL4B
Impure Ep
Immunization
Antisera
Immunoaffinity Purification by Goat-Anti-Rabbit Igs
Antibody Purification on Wheat Germ Lectin-Sepharose Ep (purified)
Anti-I
Formation of Sepharose-(Anti-I) Col.
Sepharose-Anti-I Col. (RIAC)
RIAC-purified Ep
DIAC purified Ep
Lectin Affinity Chromatography on Con-A-Sepharose 4B
Lectin Affinity Chromatography on Wheat Germ Lectin-Sepharose 6MB
Adsorption Chromatography on Hydroxylapatite
Gel Filtration on Sephadex G-100
Purified Ep
Anti-Ep
Formation of Sepharose-(Anti-Ep) Column
Sepharose-Anti-Ep Column (DIAC)

The DIAC-RIAC purified Ep can be tested for homogeneity by attempting further purification using conventional purification techniques (preferably chromatographic techniques and/or gel filtration), and assayed for biological activity.

The DIAC-RIAC purified Ep is further tested for homogeneity and characterized by electrophoretic techniques, such as gel electrophoresis, isoelectric focusing, and disc electrophoresis in non-dissociating systems according to well-known methods described by: (a) Laemmli, U.K.: Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T_4, Nature 227: 680-685, 1970; (b) Catsimpoolas, N. et al (Ed.); Biological and Biomedical Application of Isoelectric Focusing, New York, Plenum Press, 1977, and (c) Davis, B.J.: Disc Electrophoresis-II: Method and Application to Human Serum Protein, Ann. N.Y. Acad. Sci. 121:404-427, 1964.

The following examples serve further to illustrate the present invention, but not to limit its scope.

Materials: Phenyl-Sepharose CL4B, ConA-Sepharose 4B, Wheat germ Lectin-Sepharose 6MB, CNBr Activated Sepharose 4B Sephadex G100 were obtained from Pharmacia Laboratories, Inc., (Piscataway, New Jersey). Guanidine hydrochloride (ultra-pure) was obtained from Schwartz-Mann Biochemicals (Spring Valley, New York). Ethylene glycol and N-acetylglucosamine were from Sigma Chemical Company (St. Louis, Missouri). All other chemicals were from Fisher Scientific Company (Fairlawn, New Jersey), except when otherwise specifically indicated.

Concentration of all column eluates was carried out at 4°C using an Amicon ultrafiltration apparatus with YM10 membrane unless otherwise specified.

APPENDIX B

Monoclonal Anti-Ep

Ep is a weak immunogen. Accordingly, availability of pure Ep and choice of the initial immunization procedure (animals, sites and schedules) may substantially affect the efficiency of hybridoma production and the ability of the hybridoma to reliably secrete monoclonal Anti-Ep having the requisite properties.

Human Ep isolated from urine of anemic patients by Hydrophobic Interaction Chromatography on Phenyl-Sepharose CL4B and subsequently purified by Direct Immunoaffinity Chromatography followed by Reverse Immunoaffinity Chromatography (as described in the Ep Purification Patent Application) is used as the immunogen. The purity of the thus purified Ep was compared to that of homogeneous Ep provided by another investigator. The results are shown in Figure 1. The material used in this work as the antigen also shows a single band by gel isoelectric focusing and by electrophoresis under nondissociating conditions as described fully in the Ep Purification Patent Application.

In vitro immunization and subsequent fusion resulted in unstable hybrids secreting IgM. The combination of *in vivo* primary injection and *in vitro* boosting also resulted in unstable hybrids.

In vivo immunization is, therefore, preferred. Female laboratory mice are preferably used. Multiple injections in multiple sites increase the probability of obtaining a satisfactory immune response. Immune response is assayed, preferably, by Solid Phase Radioimmunoassay (SPRIA) according to the procedure of Klinman, N.R. "*The Mechanism of Antigenic Stimulation of Primary and Secondary Clonal Precursor Cells*" J. Exp. Med. 135:241-260 (1972), as set forth in detail in Example 2, below.

The Ep-neutralizing ability of immune mouse serum is preferably assayed by the *in vivo* exhypoxic polycythemic

mouse method, as described in the Ep Purification Patent Application, as said method is the most reliable (though also the most expensive and time-consuming). The mice with an acceptable immune response are selected. Immune response is measured by Anti-Ep titer and Ep-neutralizing titer. For the response to be considered acceptable, Anti-Ep titer should show at least 50% binding at 1:10,000 serial dilution (Figure 1A) and Ep-neutralizing titer should show neutralization of over 100 Ep units/ml of mouse serum. These performance characteristics are rather formidable, considering the weak immunogenic properties of Ep. Accordingly, the Ep used for immunization should be the purest possible and the number of mice immunized should be relatively large. Generally, assuming careful selection and execution of the immunization protocol, about one mouse in six immunized will exhibit an acceptable immune response.

Hybridomas are fused from spleen lymphocytes of the mice showing acceptable immune response and from non-secreting mouse myeloma cells. NS-1, a non-immunoglobulin secreting myeloma cell line of Balb/c origin, commercially available (from Mutant Cell Repository, Institute of Medical Research, Camden, N.J.) and resistant to 8-azaguanine is preferred. Other types of myeloma cell lines that, in principle, would also be suitable include those discussed in Kohler, G., Howe, S.C., and Milstein, K.; Fusion Between Immunoglobulin-Secreting and Non-Secreting Myeloma Cell Lines, Eur. J. Immunol. 1976, 6:292-295.

Fusion is carried out according to the general procedure of Kennett, R.H. in Monoclonal Antibodies, Hybridomas: A New Dimension in Biol. Analyses (Kennett et al, Eds.) Plenum Press, 365-367 (1980), with such modifications as are set forth in Example 2, below.

Fusion hybrids are fed and grown in selective media. Surviving hybridoma cells from these media are propagated in culture and their culture media are screened for the presence of Anti-Ep by solid phase radioimmunoassay

(SPRIA) in accordance with the method of Klinman, supra. Positive hybridoma cultures are then assayed for Ep-neutralization, preferably by the exhypoxic polycythemic mouse method.

After Ep-binding and/or Ep-neutralization have been confirmed, the positive hybridoma cultures are immediately cloned and grown in liquid media. Immediate cloning and recloning are necessary to ensure hydridoma stability. It is customary in the art not to consider a hybridoma culture stable until 100% of clone colonies derived from positive cultures upon recloning are also positive. The stability of hybridoma cells of the present invention has been confirmed by prolonged storage (1 to 2 years) without decline in Anti-Ep secretion. Three stable clones were thus isolated (hereinafter designated as 7A7, 7B9, and 2A10) from 6460 hybridomas in a total of 10 fusions.

After successful cloning of Anti-Ep-secreting hybridomas, large quantities of high-titered antibodies can be obtained by growing hybridomas in culture, or, preferably, by ascites induction. Ascites tumor can be induced by intraperitoneal injection of cloned hybridoma cells into pristane primed syngeneic mice (about 10^7 hybridoma cells per primed mouse). After ascites has been allowed to develop for several (preferably 2-3) weeks, ascitic fluid is harvested from the peritoneal cavity (multiple collections are possible and desirable, since the objective is to obtain as much hybridoma as possible). Monoclonal Anti-Ep activity of ascitic fluid is tested by SPRIA.

In order to confirm that the ascites Anti-Ep activity is in fact due to immunoglobulin, and in order to prepare stable antibody preparations, free of degradative enzymes such as proteases and nucleases, suitable for Ep-purification, for Ep mRNA identification and screening of an Ep-cDNA library, the ascites immunoglobulin must be purified. Ascites immunoglobulin and its subclass are

characterized to help design and develop the most effective purification procedures.

The types of immunoglobulin chains are characterized, preferably by use of an isotype-specific rabbit Anti-mouse immunoglobulin kit (Boehringer-Mannheim, Indianapolis, Indiana). This test is also used to confirm the results after immunoglobulin purification.

The types of the three antibodies corresponding to the three stable clones produced in this work were: IgG2a/k (from 7A7), and IgG1/k (from 7B9 and 2A10).

The antibody purification technique was subject to optimization, as many of the available methods presented serious drawbacks.

Protein A-Sepharose affinity chromatography is generally satisfactory for the purification of IgG2a/k. However, this affinity adsorbent fails to bind IgG1 efficiently (even at a slightly basic pH such as 8.4). Ammonium sulfate fractionation, followed by diethylaminoethyl cellulose (DEAE-cellulose) ionic exchange and Sephadex G-200 gel filtration, fails to remove all contaminating proteases and nucleases. These degradative enzymes are deleterious to the antibodies, to Ep activity (during immunoaffinity chromatography purification using monoclonal Anti-Ep as the adsorbent) and to Ep-mRNA stability (during purification by polysome immunoprecipitation). DEAE-Affi gel blue chromatography does remove protease and RNase effectively but is difficult to optimize (maximum resolution conditions have to be determined separately for each antibody) and often results in low yields. Protein A-Sepharose CL4B affinity chromatography followed by precipitation of the eluted IgG2a with ammonium sulfate, is used for purification of monoclonal Anti-Ep 7A7. The yield is usually at least 90%. Affinity purified goat anti-mouse immunoglobulin covalently coupled to Sepharose 4B is effective for IgG1 purification and remove protease and RNase. It can thus be used for the purification of monoclonal Anti-Ep 7B9 and

Anti-Ep 2A10 (IgG1/k). Both monoclonal preparations can thus be free of detectable degradative enzyme activity. The thus recovered purified immunoglobulins can be quantitated by SPRIA (by measuring their native Ep-binding ability) and their heavy and light chains can be characterized by SDS-PAGE. Ep-neutralization ability can be tested in vivo, by exhypoxic polycythemic mouse bioassay. Preferably, native Ep binding is determined by immunoblot and ^{125}I-Ep (denatured) binding is determined by immunoprecipitation.

Hybridoma 7A7 is a much stronger Anti-Ep producer than either 7B9 or 2A10, secreting more than ten times as much antibody. Of the three antibodies isolated in this work, only 2A10 neutralizes Ep. All three antibodies recognize (bind to) both native and ^{125}I-labeled Ep.

The difference in the binding affinity between the monoclonal antibodies of the present invention and the antibody reported by Weiss, et al., supra, is illustrated by the fact that, in competitive radio-immunoprecipitation of ^{125}I-Ep with unlabeled Ep, a ratio of native to labeled Ep of 41 was sufficient to cause 50% inhibition of ^{125}I-Ep binding, (as opposed to a ratio of 2500 reported in Weiss et al). The increased binding affinity of the antibodies of the present invention is extremely significant because it permits the non-neutralizing antibodies disclosed herein to be used in Ep purification by immunoaffinity chromatography using purified monoclonal Anti-Ep as the immunoadsorbent. Immunoglobulin from 7A7(IgG2a) is preferred because it is secreted in larger quantities, it can be effectively purified (with a high yield), and it does not neutralize (and thus possibly inactivate) Ep.

Purification of Ep by immunoaffinity chromatography (using monoclonal Anti-Ep from 7A7 as the immunoadsorbent) yields, in one step, Ep of purity and yield comparable to those obtained with a combination of DIAC and RIAC. Furthermore, since these monoclonal antibodies also

recognize Ep from other mammals such as sheep, rabbit, rat and mouse, they would also be very useful in affinity purification of Ep from those and other species.

In addition to providing improved means for Ep purification, the production and purification of monoclonal Anti-Ep according to the present invention is expected to facilitate studies on erythropoiesis. The monoclonal Anti-Ep of the present invention provides useful probes for detection of Ep-synthesizing cells, Ep-responsive cells and Ep specific receptors, and can be used advantageously in conjunction with immunofluorescence and radioimmunoassay techniques. The regulation of Ep-gene expression under both normal and pathological states can be studied by the identification of Ep-mRNA and its translation product using immunoblotting, immunoprecipitation and immunoautoradiography. Moreover, the monoclonal antibodies of the present invention are useful probes in the cloning of Ep-gene and in the identification of the cloned Ep-gene product. Many of the above uses of monoclonal Anti-Ep have been confirmed in practice, as more fully set forth in the Ep Clone Patent Application.

The present invention is further described in the following Examples which are intended to illustrate it but not to limit its scope.

Materials and Sources:

Fetal Calf Serum (FCS) and tissue culture media were from Gibco, Grand Island, N.Y.

Sodium azide was from Sigma, St. Louis, Mo.

Bovine serum albumin (BSA) was from Schwartz Mann, Spring Valley, N.Y.; and

$^{59}FeCl_3$ was from New England Nuclear, Boston, Mass.

The Antigen: Human Ep was isolated from urine of anemic patients by Hydrophobic Interaction Chromatography on Phenyl-Sepharose CL4B and subsequently purified by Direct and Reverse Immunoaffinity Chromatography (DIAC-RIAC).

What is claimed is:

1. A peptide immunochemically reactive with a monoclonal antibody to human erythropoietin.

2. A peptide according to claim 1, produced by an organism, containing at least one DNA fragment comprising a DNA sequence coding for said peptide.

3. A peptide according to claim 2, wherein said organism has been transformed by an expression vector comprising said DNA fragment.

4. A fusion protein produced by an organism, said protein consisting essentially of a peptide immunochemically reactive with a monoclonal antibody to human erythropoietin and a portion of an endogenous protein of the organism.

5. A DNA fragment having a deoxynucleotide sequence encoding a peptide immunochemically reactive with a monoclonal antibody to human erythropoietin.

6. A recombinant DNA molecule comprising a DNA fragment according to claim 5.

7. An organism transformed by a recombinant DNA molecule, according to claim 6.

8. An organism according to claim 7 comprising a bacterium of the species Escherichia coli.

9. A recombinant DNA molecule according to claim 6 containing said DNA fragment at a site within said molecule suitable for expression of the peptide coded for by said DNA fragment.

10. A recombinant DNA molecule according to claim 9 wherein said peptide is capable of expression as a fusion protein.

11. A recombinant molecule according to claim 6 wherein said recombinant DNA molecule is a hybrid plasmid derived from pBR322.

12. A recombinant molecule according to claim 11 wherein said DNA fragment is inserted in the Providencia stuartii I cleavage site of said plasmid.

13. A recombinant DNA molecule according to claim 12, wherein said DNA fragment is inserted by homopolymeric dC:dG tailing.

14. A functional mRNA molecule carrying human erythropoietin message, in purified form.

15. A functional mRNA molecule according to claim 14, derived from a renal carcinoma cell, said renal carcinoma having a high erythropoietin titer.

16. A functional mRNA molecule according to claim 15, wherein said titer is greater than about 1 unit/g of tissue.

FIG. 1

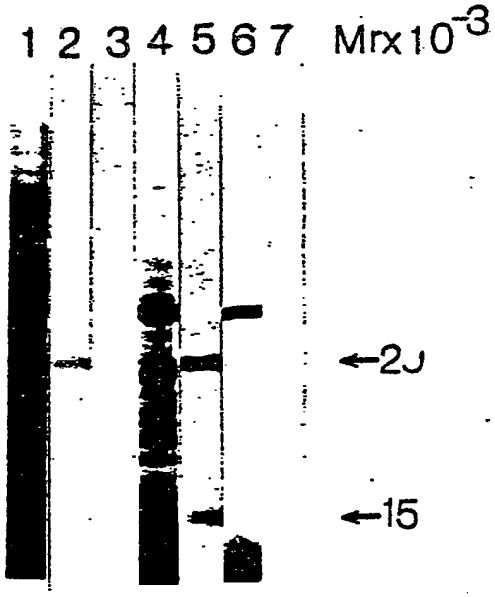


FIG. 2

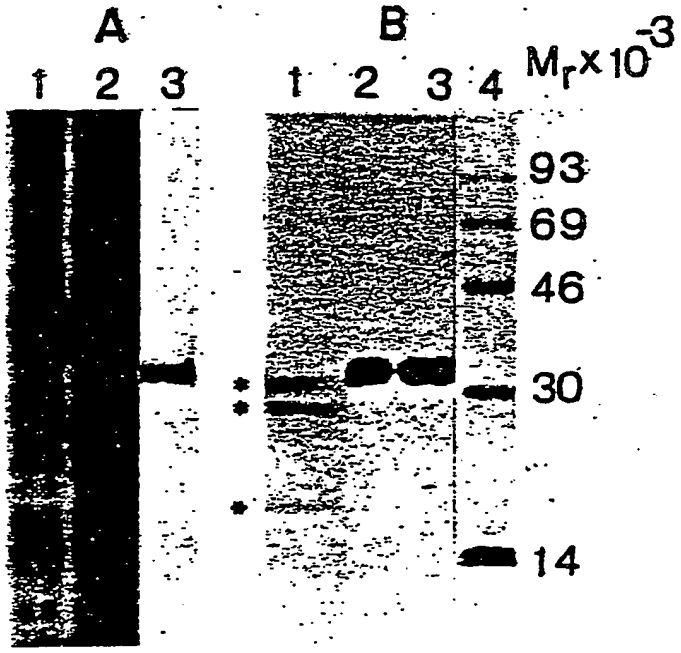
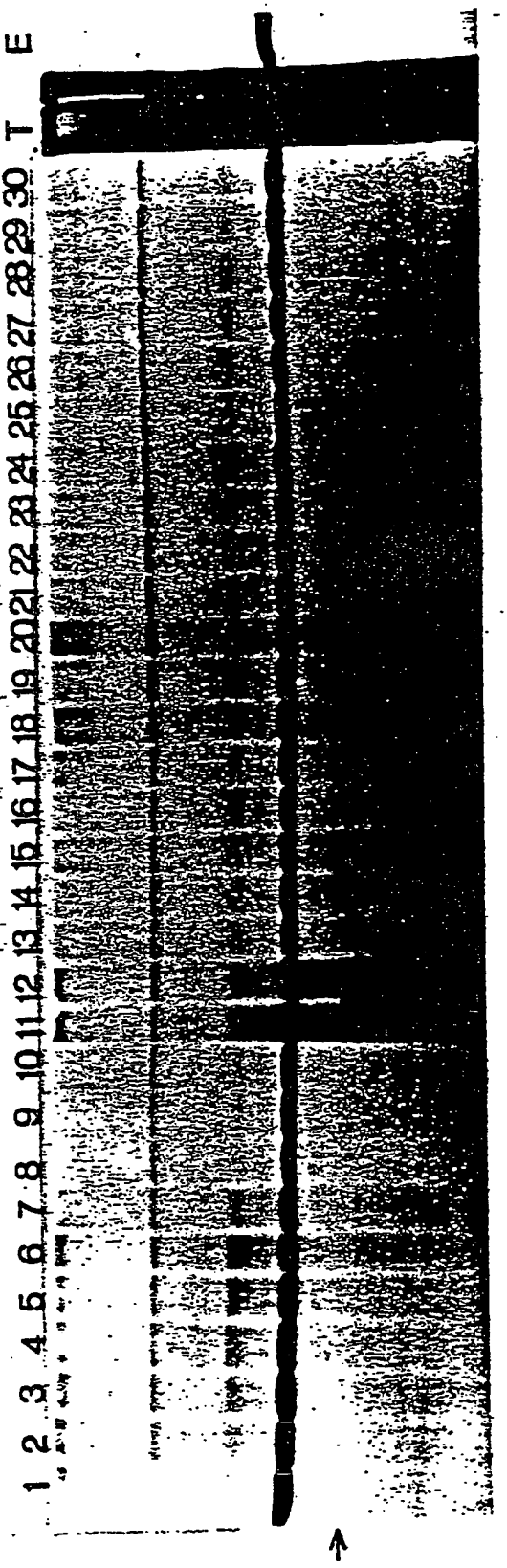


FIG. 3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30 29 28 27 26 25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

FIG. 4

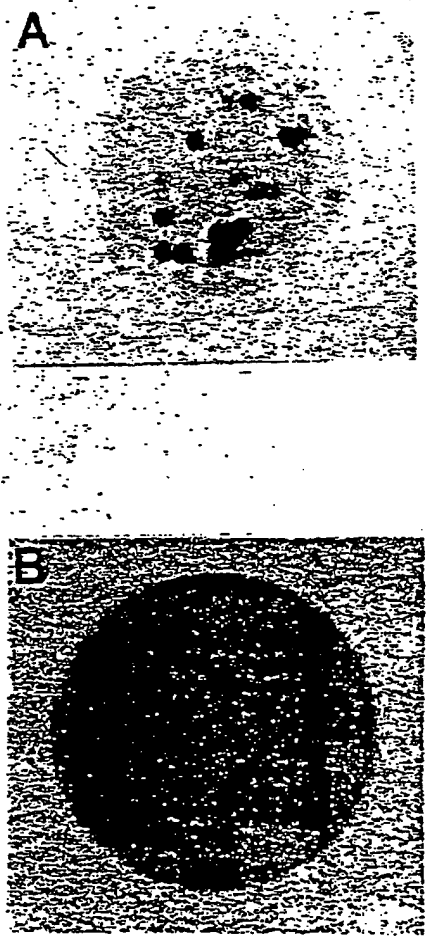


FIG. 5

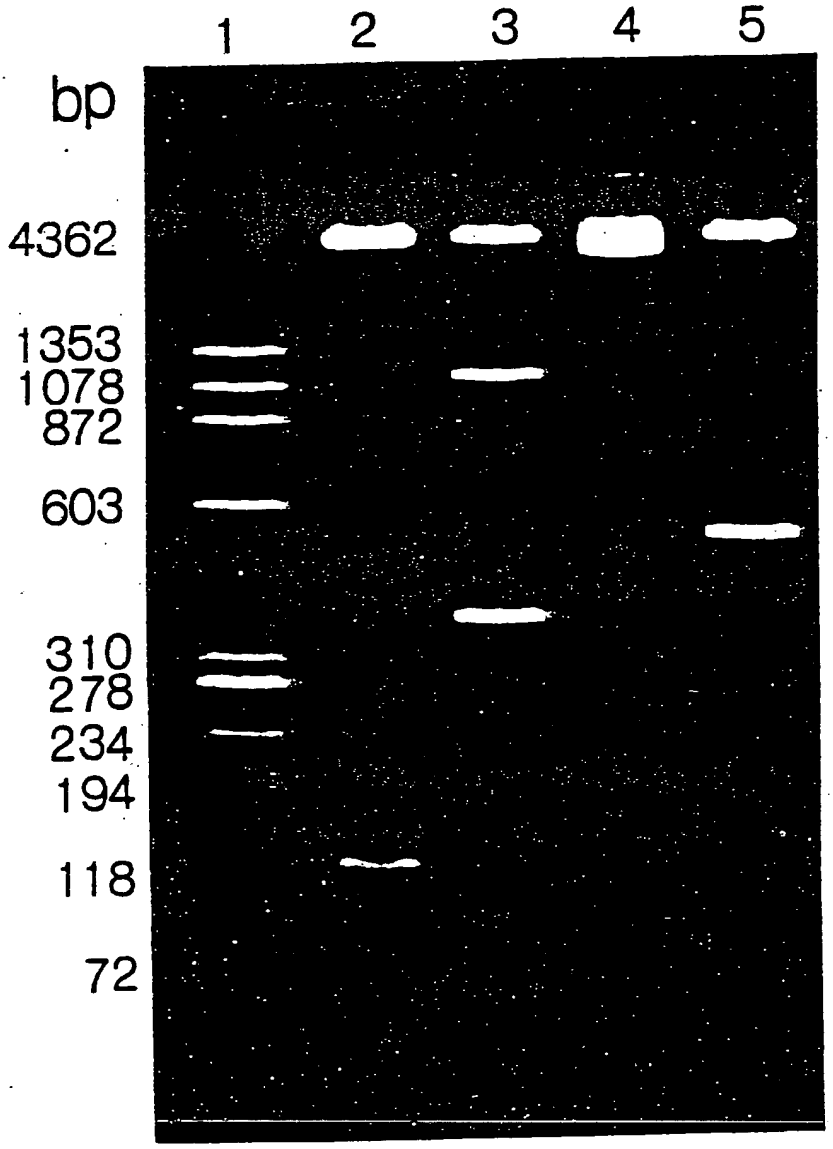


FIG. 6A

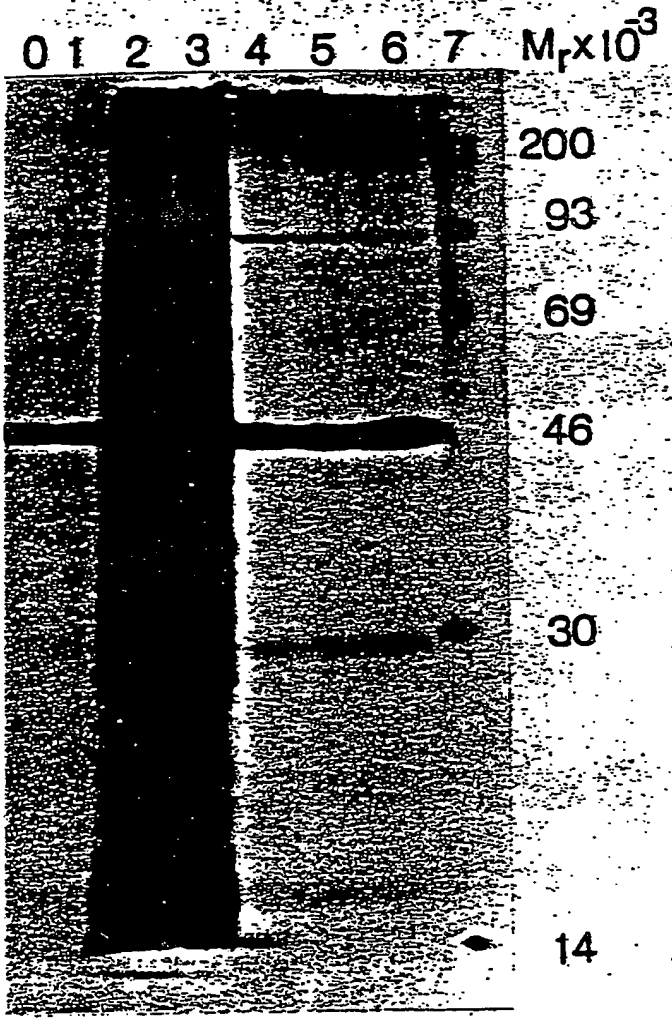


FIG. 6B

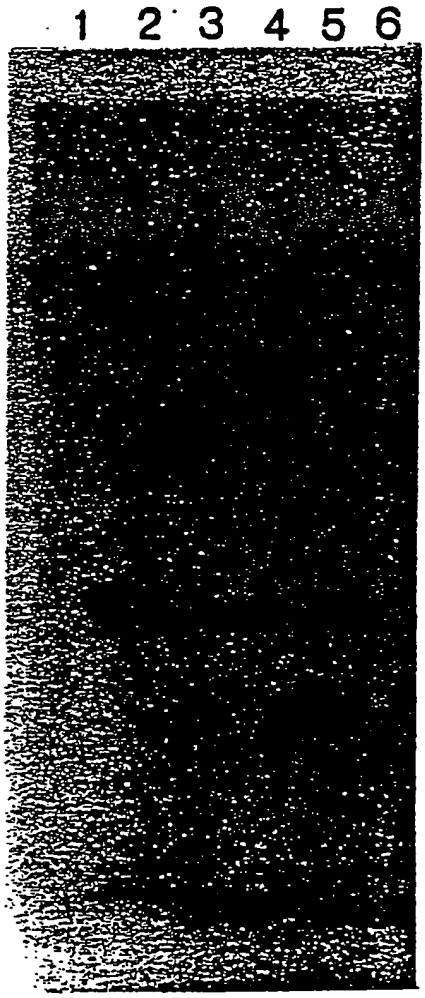
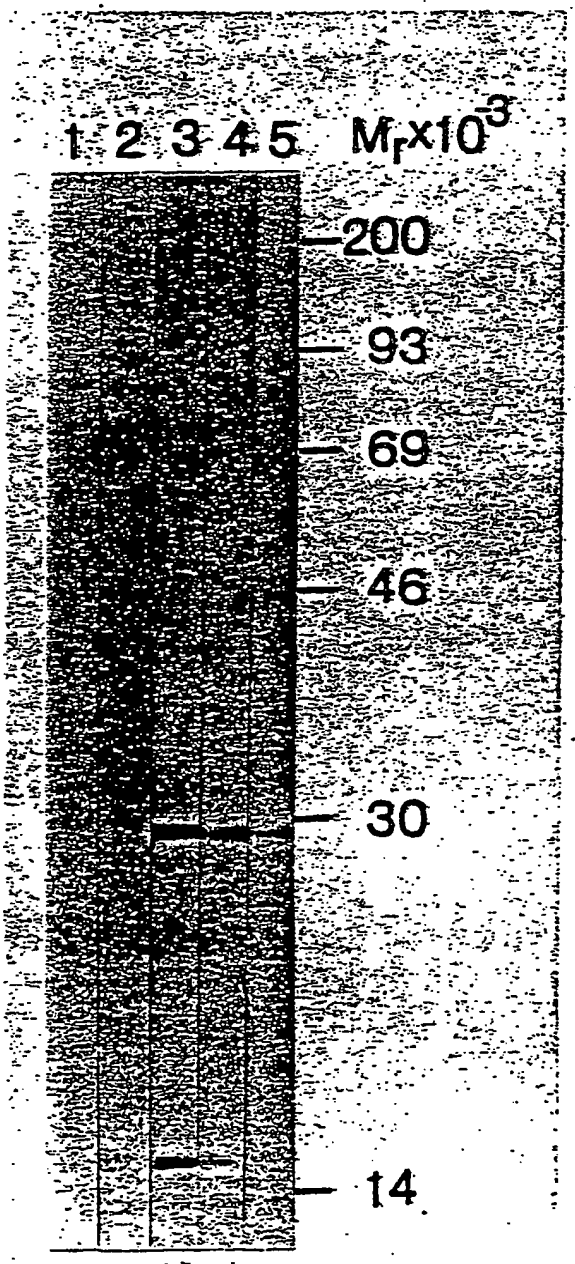


FIG. 7



INTERNATIONAL SEARCH REPORT

International Application No PCT/US85/00054

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC [4]

260/112, 536/27, 435/172.3
C07F 15/12 C12N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
435	68, 172.3, 317
536	27
260	112

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Computer Search-Biosis; Chemical Abstract Index

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
T	Lee-Huang 1984 "Cloning and Expression of Human Erythropoietin cDNA in E scherichia Coli", Proc. Natl. Acad. Sci. V 81 p2708-12	1-16
Y	Lee-Huang 1982 (Abstract) "Monoclonal Antibodies to Human Erythropoetin" Fe. Proc. V 41 p520	1-16
X	Lin et al 1984. (Abstract) "Cloning of the Monkey Erythropoietin Gene" J. Cell. Biochem (Supp 8B) p45	5-16
Y	UK Application 2,085,887 6 May 1982 Hayashibara et al "Process for the Production of Human Erythropoietin"	1-4
Y	US 4,377,513 22 Mar 1983 Sugimoto et al. "Process for the Production of Human Erythropoietin"	1-4

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [3]	Date of Mailing of this International Search Report [3]
May 6, 1985	07 JUN 1985
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Joanne M. Giesser

Form PCT/ISA/210 (second sheet) (October 1981)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	US 4,503,151 Paddock et al 5 Mar. 1985 "Recombinant cDNA Construction Method and Hybrid Nucleotides useful in Cloning"	5-16

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

Claims 1-4 drawn to a peptide

Claims 5-6 drawn to DNA, RNA, and microorganism

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☒ No protest accompanied the payment of additional search fees.